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Interaction between IDH1 WT and calmodulin and its implications for glioblastoma cell growth and migration

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ABSTRACT

Isocitrate dehydrogenase (IDH) mutations are found in low-grade gliomas, and the product of the IDH mutant (MT), 2-hydroxyglutarate (2-HG), is the first known oncometabolite. However, the roles of the IDH wild type (WT) in high-grade glioblastoma, which rarely has the IDH mutation, are still unknown. To investigate possible pathways related to IDH WT in gliomas, we carried out bioinformatics analysis, and found that IDH1 has several putative calmodulin (CaM) binding sites. Pull-down and quantitative dissociation constant (Kd) measurements using recombinant proteins showed that IDH1 WT indeed binds to CaM with a higher affinity than IDH1 R132H MT. This biochemical interaction was demonstrated also in the cellular environment by immunoprecipitation with glioblastoma cell extracts. A synthetic peptide for the suggested binding region interfered with the interaction between CaM and IDH1, confirming the specificity of the binding. Direct binding between the synthetic peptide and CaM was observed in an NMR binding experiment, which additionally revealed that the peptide initially binds to the C-lobe of CaM. The physiological meaning of the CaM-IDH1 WT binding was shown with trifluoperazine (TFP), a CaM antagonist, which disrupted the binding and inhibited survival and migration of glioblastoma cells with IDH1 WT. As CaM signaling is activated in glioblastoma, our results suggest that IDH1 WT may be involved in the CaM-signaling pathway in the tumorigenesis of high-grade gliomas.

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1. Introduction

Isocitrate dehydrogenase (IDH) catalyzes the oxidative decarboxylation of isocitrate, yielding alpha-ketoglutarate and CO_2. The reaction, which involves NAD(P)H as a coenzyme, is reversible under physiological conditions. IDH has drawn considerable interest, as its mutation, particularly R132 H mutation (MT) in IDH1, has been found associated with brain cancer [1]. Initially, IDH1 was suggested to be a tumor suppressor, and its mutant form (IDH1

MT) was reported to suppress the activity of wild-type IDH1 (IDH1 WT) [2]. However, IDH1 MT was soon shown to produce 2-hydroxyglutarate (2-HG) through its neomorphic activity [3], 2-HG becoming known as the first oncometabolite. Since then, much research has been carried out to establish the causal roles of IDH MT and 2-HG in glioma tumorigenesis [4,5]. However, direct tumorigenic roles have not been firmly established in glioma [6], though an inhibitor of IDH1 MT has been developed as a drug for use in acute myeloid leukemia (AML) with IDH1 mutation [7]. Interestingly, IDH1 mutations have been found mainly in low-grade (grade I and II) or secondary glioma, and quite rarely in high-grade primary gliomas (grade IV), including glioblastoma multiforme (GBM) [8]. Furthermore, the wild-type IDH allele seems to be required for the high levels of 2-HG found in glioma with IDH MT [9]. Therefore, most research on the relationship between IDH and glioma has focused on IDH MT and low-grade gliomas, and relatively little is known on the roles of IDH WT in more malignant high-grade gliomas.

Calmodulin (CaM) is a well-known Ca^{2+}-binding protein that mediates various cell-signaling pathways by interacting with its targets. CaM's level is higher in malignant GBM than in normal brain or low-grade glioma, and its expression levels are strongly correlated with glioma cell's migration capabilities [10]. Higher expression of CaM is associated with poorer prognosis and shorter survival in glioma patients [10]. Several aspects of CaM-signaling have been correlated with growth and migration of gliomas. [11,12]. CaM-dependent kinase II (CaMKII) binds and regulates CLC-3, a chloride channel elevated in grade IV glioma, and pharmacological inhibition of CaMKII reduces glioma cell migration [11]. CaM itself binds K-ras [13,14], whose signaling has been found to be important in glioblastoma maintenance [15]. Disruption of CaM-K-ras interaction might have a potential role in glioblastoma, as CBP501 that binds CaM and blocks the interaction is in clinical trials for non-small-cell lung cancer [16,17]. Actually, trifluoperazin (TFP), an anti-schizophrenic drug with well-known CaM antagonist activity, has exhibited anti-glioblastoma effects both *in vitro* and *in vivo* [18]. Therefore, CaM might interact with other proteins, thereby affecting the growth, migration and malignancy of glioblastoma. In this study, we investigated the direct interaction between CaM and IDH1 WT, known to be correlated with malignant glioma, and its implications for glioma growth and migration.

2. Materials and methods

2.1. Protein expression and purification

Purified CaM protein was obtained as described before [19]. IDH1 WT and R132H MT proteins were prepared as described in detail in Supplementary Information. All of the protein samples were treated with 5 mM EGTA at pH 8.0 to remove inherently bound Ca^{2+}.

2.2. Pull-down assay

Immobilized CaM resin was prepared using purified CaM and NHS-activated sepharose 4 fast resin (GE Healthcare Life Sciences), as described previously [19]. Detailed methods are described in Supplementary Information.

2.3. Co-immunoprecipitation

U87 cells expressing IDH1 WT and R132H MT [20] were applied to the co-immunoprecipitation method according to the manufacturer's protocol. Procedures in detail are described in Supplementary Information.

2.4. Surface Plasmon Resonance (SPR)

The protein interaction was analyzed by Reichert SR7500DC spectrometry (Reichert Technologies, Depew, NY, USA) following the manufacturer's instructions. Detailed procedures are described in Supplementary Information.

2.5. NMR

^{1}H-^{15}N heteronuclear single-quantum coherence (HSQC) spectra were obtained on an 800 MHz spectrometer (Bruker, Billerica, MA, USA) equipped with a cryogenic probe. Additional information is provided in Supplementary Information.

2.6. Scratch-wound-healing assay

U87 cells over-expressing IDH1 WT were treated for scratch-wound-healing assay. Detailed methods are described in Supplementary Information.

2.7. Cell viability and proliferation assay

Cell viability was assessed using an MTT assay, and proliferation was measured using crystal violet staining. Both assays are well-established, and were performed using standard protocols. Detailed procedures are described in Supplementary Information.

3. Results

3.1. Prediction of binding between CaM and IDH1.

To find possible binding targets of IDH relevant to its involvement in glioma severity, we analyzed its sequence for the presence of protein-interaction sites. The analysis with Scansite [21] presented possible kinase interaction and modification sites. Among them, we took notice of CaM-dependent kinase 2 (Fig. 1A), particularly as a recent paper suggested a relationship between CaM signaling and IDH1 mutant status in glioma [22]. We then further analyzed the IDH1 sequence for the presence of possible CaM-binding sites, as the requirements for CaM-binding are well established (Fig. 1B). Interestingly, amino acids between G339 and L346 in IDH1 exhibited a stretch of very high probability of CaM binding, with 8 consecutive amino acids showing scores of 9. There were no other regions with such a high binding score. The analysis suggested that there might be specific binding between IDH1 and CaM involving this region (see again Fig. 1B).

3.2. Biochemical assay for binding between CaM and IDH1 WT/MT.

To test the suggested interaction between CaM and IDH1 experimentally, we prepared recombinant proteins for both CaM and IDH1. For IDH1, we made both wild-type (WT) and mutant (R132H MT) proteins, since they are associated with different clinical outcomes of glioma [1]. In addition, CaM signaling (i.e.CaMKK2 expression) was shown to differ between IDH1 WT and MT glioma cells [22]. The bindings were monitored by pull-down assay employing resins with immobilized CaM (Fig. 1C). Neither IDH1 WT nor MT bound to sepharose resin without immobilized CaM, but more WT bound to the CaM-sepharose than R132H. The absence of BSA binding to CaM-immobilized resin excluded possible non-specific binding effects. The binding was weaker in the presence of salt (100 or 150 mM), but IDH1 WT still exhibited significant binding, whereas R132H did not (Fig. S1). To investigate the binding interaction more quantitatively, we also obtained the dissociation constant (Kd) using surface plasmon resonance (SPR). Consistent with the above pull-down and immunoprecipitation, IDH1 WT exhibited an order-of-magnitude-higher affinity than R132H MT toward CaM, the Kd values being 2.38×10^{-7} M and 2.33×10^{-6} M, respectively (Fig. 1D).

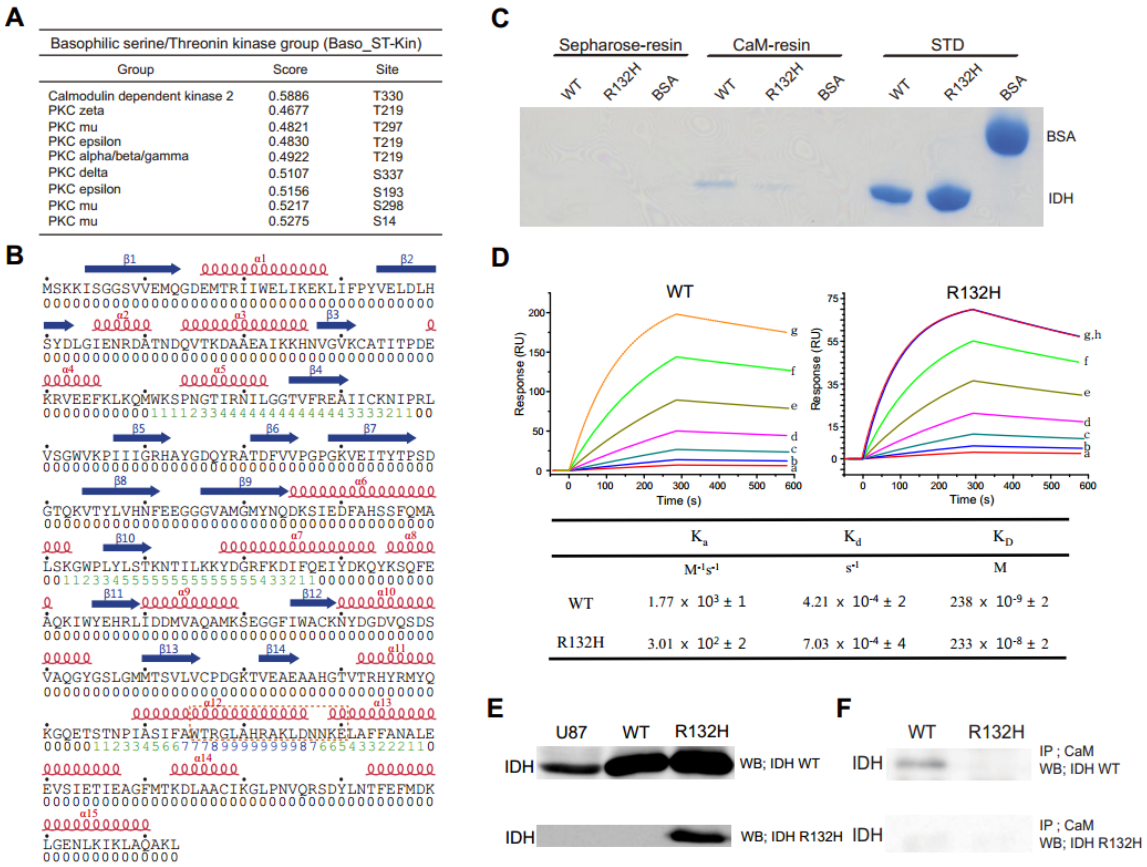

Figure 1. Bioinformatic sequence analysis of IDH1 and identifications of the binding between of IDH1 proteins with CaM. (A) Motif scan results of IDH1. Signaling domains of IDH1 was analyzed using Scansite program, and the predicted kinase interaction and modification motifs are shown. (B) Predicted CaM-binding sites of IDH1. The numbers represent the probability of CaM binding. The putative binding site is boxed as dotted orange lines. (C) Pull-down assay for the binding between of IDH1 proteins with CaM. Resin-immobilized CaM was incubated with recombinant IDH1 WT and R132H MT. Sepharose CL-4B resin without immobilized CaM was used as the negative control. BSA was used to test the non-specific binding. Input shows the molecular sizes of the proteins. (D) Quantitative binding analysis for the CaM-IDH1 interaction using Surface Plasmon Resonance (SPR). Responses upon the injection of the analytes (IDH1 WT/R132H MT) are presented (RU; arbitrary unit). Binding affinity were calculated as K_D 238 ± 2 nM (WT) and 2330 ± 2 nM (R132H). (E-F). Co-immunoprecipitation of CaM and IDH1 proteins in WT/R132H-expressing U87 cells. (E) Specificities of antibodies against IDH1 WT and R132H MT were tested using the lysates from parental (U87), IDH1 WT, and R132H MT U87 cells. (F) The cell lysates were co-immunoprecipitated using CaM antibody and protein A/G agarose and blotted with antibodies against IDH1 WT and R132H.

3.3. Binding of CaM and IDH1 in a cellular environment.

To test IDH1-CaM binding in an actual cellular environment, IDH1 WT and MT proteins were heterologously expressed in U87 glioma cells and the binding with endogenous CaM was tested by immunoprecipitation. The glioma cell line was chosen because IDH1 mutation status greatly affects glioma prognosis, IDH1 MT being associated with better prognosis [1]. The two proteins were co-immunoprecipitated with CaM antibody, and then a Western blot was carried out using antibodies specific to either IDH1 WT or R132H MT (Fig. 1E-F). As shown in Figure 2D, binding was observed between IDH1 WT and CaM, but not between IDH1 MT and CaM. The data confirmed binding between

IDH1 and CaM in the native cellular environment and its dependency on mutation status.

3.4. Confirmation of the binding site.

Having confirmed the binding between CaM and IDH1 WT, we investigated whether the binding region predicted above is actually involved in the interaction. To that end, we synthesized a peptide corresponding to the site and flanking region with the sequence WTRGLAHRAKLDNNKE. In the three-dimensional structure of IDH1 (PDB ID: 1T0L), the peptide was located in a region including two helices with a loop between them. The site also turned out to be surface-exposed and, thus, available for binding interaction (Fig. 2A). The actual binding of this peptide to CaM was tested with a pull-down competition assay. The addition of the peptide to the CaM-IDH1 mixture significantly decreased the binding between the two proteins (Fig. 2B). To confirm that the binding between the peptide and CaM is direct, NMR spectroscopy was employed with ^{15}N-labeled CaM. The addition of the excess peptide (2 mM) to CaM (200 μM) induced readily visible changes in the CaM spectrum (Fig. 2C). At an equimolar concentration (200 μM), the spectral changes were smaller. Importantly, this NMR experiment also provided the information on the CaM residues involved in the peptide-binding, as the interaction leads to peak changes (Fig. 2D). When mapped onto the three-dimensional structure of CaM, those residues were found in a small patch on the C-lobe (Fig. 2E) of CaM, which suggests that CaM-IDH1 binding occurs, at least initially, through the C-lobe. The results also suggest that CaM-IDH1 binding is calcium-independent, as the spectra were acquired in the presence of EGTA. Overall, the biochemical and spectroscopic binding experiments showed that CaM-IDH1 binding is direct, is mediated by the suggested peptide region, and is calcium-independent.

3.5. CaM antagonist TFP inhibits CaM-IDH1 WT interaction and reduces migration of IDH1-WT-over-expressed U87 cells.

After confirming the interaction of CaM with IDH1 WT, and the much weaker interaction with IDH1 MT, we investigated the functional aspects of the interaction. We first tested the effects of a functional CaM antagonist, TFP, on the binding between the two proteins. To determine if this antagonist can inhibit CaM-IDH1 binding, we performed a pull-down assay in the presence of TFP. The results showed that CaM-IDH1 WT binding is indeed inhibited by TFP (Fig. 3A). As TFP induces major conformational changes in CaM and thereby inhibits CaM-target binding [23], our result suggests that TFP can affect the functions of CaM-IDH1 WT complex. It has been shown that glioma with IDH1 WT is much more aggressive than with IDH1 MT, and that TFP exhibits anti-metastatic activity [24] . In addition, CaM, through its interaction with p68 helicase, is involved in cancer metastasis and migration, and this interaction can be abrogated by a peptide [25]. Therefore, we tested if TFP's inhibition of the interaction between CaM and IDH1 has any implications for the migration of U87 glioblastoma cells. We performed a scratch-wound-healing assay on U87 cells transfected with IDH1 WT in the presence of TFP. As shown on Fig. 3, TFP inhibited the migration of U87 cells expressing IDH1 WT (Fig. 3B-C). The TFP's effect on cell proliferation was also tested using crystal violet staining, and the result shows that TFP inhibited the IDH1 WT cell proliferation in a concentration dependent manner (Fig. 3D). We also confirmed the effect of TFP on native U87 cells not transfected with IDH1 WT or MT genes (Fig. S2).

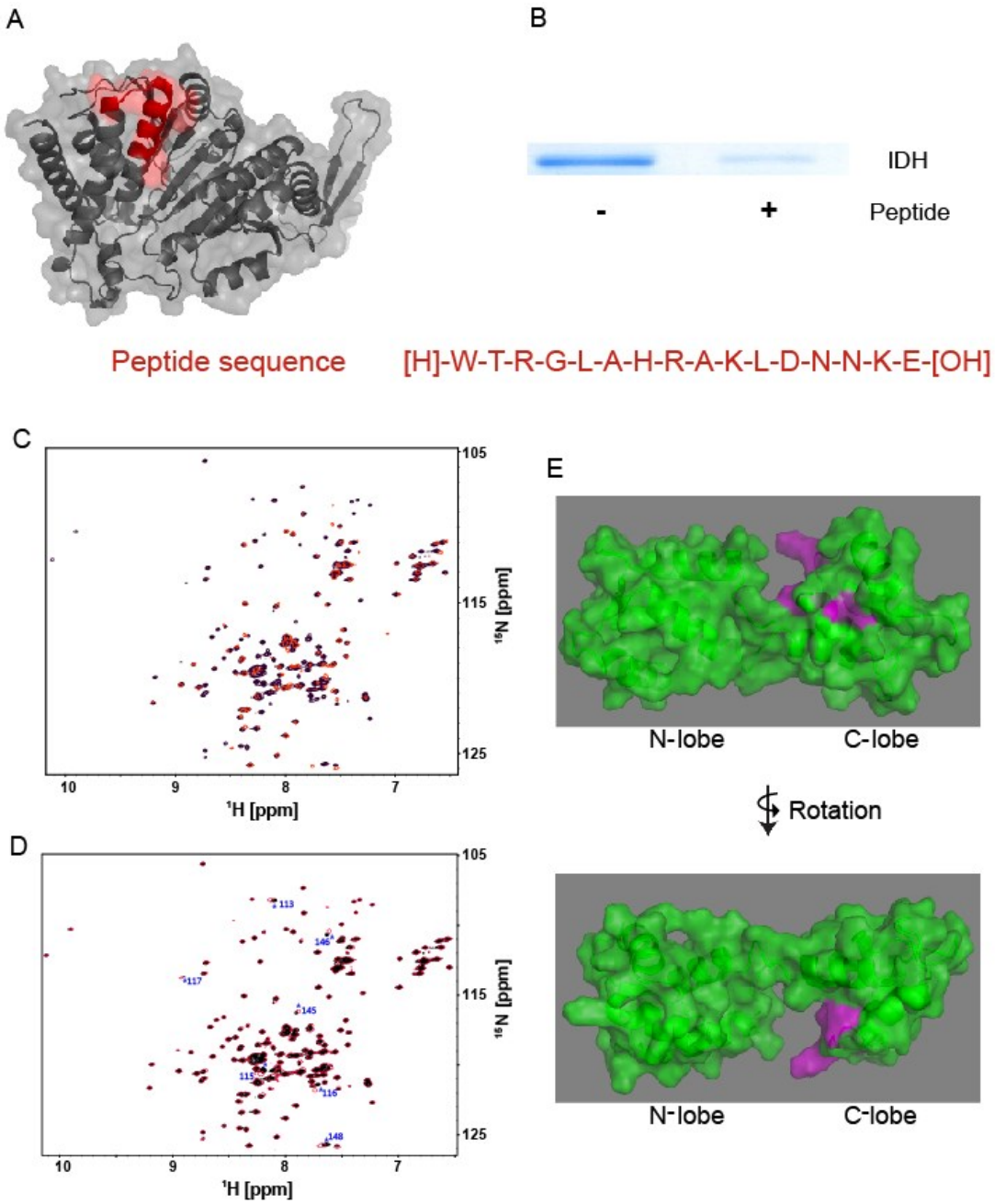

Figure 2. Binding sites for the interaction between IDH and CaM. (A) The predicted CaM-binding site on the IDH1 three-dimensional structure is shown in red (PDB ID: 1T0L). (B) The pull-down competition experiment with immobilized-CaM for the binding to IDH1 WT (0.5 mM) in the absence and presence of the IDH1 peptide (5 mM). (C-D) ^{1}H-^{15}N HSQC NMR spectrum of ^{15}N-labeled CaM (200 μM) in the absence (black) and presence of the IDH-peptide (red) (C: 2 mM; D: 200μM of IDH-peptide). (D) The CaM residues that changed upon the IDH1 peptide binding are indicated. (E) The changed residues in (D) are indicated in magenta on the CaM PDB structure (PDB ID: 1CFD).

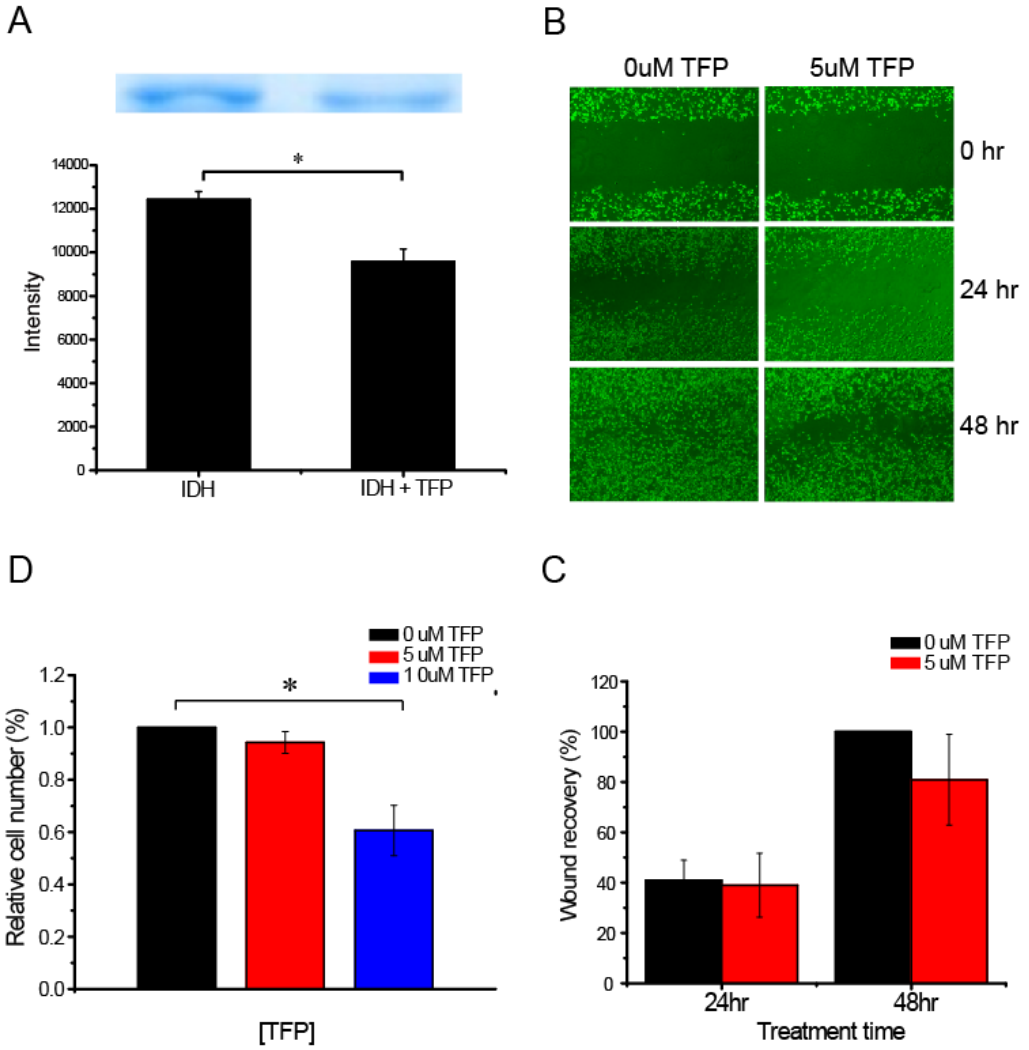

Figure 3. Effects of TFP in protein-protein interaction, cell migration and proliferation. (A) Pull-down assay for the CaM-IDH1 binding with immobilized-CaM in the presence or absence of TFP. The lower bar graph is the densitometric representation of the three independent pull-down experiments (*; $p < 0.05$). (B-C) Migration of IDH1-WT-expressing U87 cells were assessed by scratch-wound-healing assay. The cells were photographed (B) and analyzed using Image J (C). (D) Proliferation of IDH1-WT-expressing U87 cell measured by crystal violet assay. After staining with crystal violet, the relative cell numbers were evaluated with Image J program (*; $p < 0.05$).

4. Discussion

IDH1 MT has drawn considerable attention due to its neomorphic activity in generating 2-HG and its involvement in the tumorigenesis of glioma and other cancers. However, it has also been recognized that mutation is almost exclusively found in low-grade glioma and that patients with IDH1 mutation have better prognoses than those with IDH1 WT. Interestingly, the gene level of IDH1 itself is significantly higher in GBM, the most aggressive form of glioma, than in lower-grade gliomas or normal-patient samples (Fig. S3). Therefore, it seemed worthwhile to investigate the difference between IDH1 WT and MT in the context of protein interactions implicated in gliomas. Our results showed that IDH1 WT and MT have different binding activities toward CaM whose expression is associated with poorer prognosis and shorter survival of glioma patients [10].

Then, how could just one residue mutation can lead to such a significant binding affinity difference and biological function? Despite the small-scale one-residue mutation, the structural changes are propagated to distant regions with significant differences in the alignment of the secondary structural elements (Fig. 4). As is consistent with its involvement in binding, the CaM-binding site in IDH1 is located in a cavity region of IDH1. In addition, the three-dimensional conformation of the amino acid residues in the region seems to affect the accessibility of the cavity. For example, K345 in the binding site is solvent-exposed in IDH1 WT, whereas it is buried and inaccessible for binding in IDH1 MT. Also, IDH1 MT has a protruding amino acid, K350, in the cavity region, which contributes to the smaller cavity and possible inhibition of protein interaction. These differences seem to generate the larger and more accessible cavity in the CaM-binding region in IDH1 WT. Therefore, IDH1 WT can have a higher binding affinity, even though the sequences in the CaM-binding region are the same between the IDH1 WT and MT proteins.

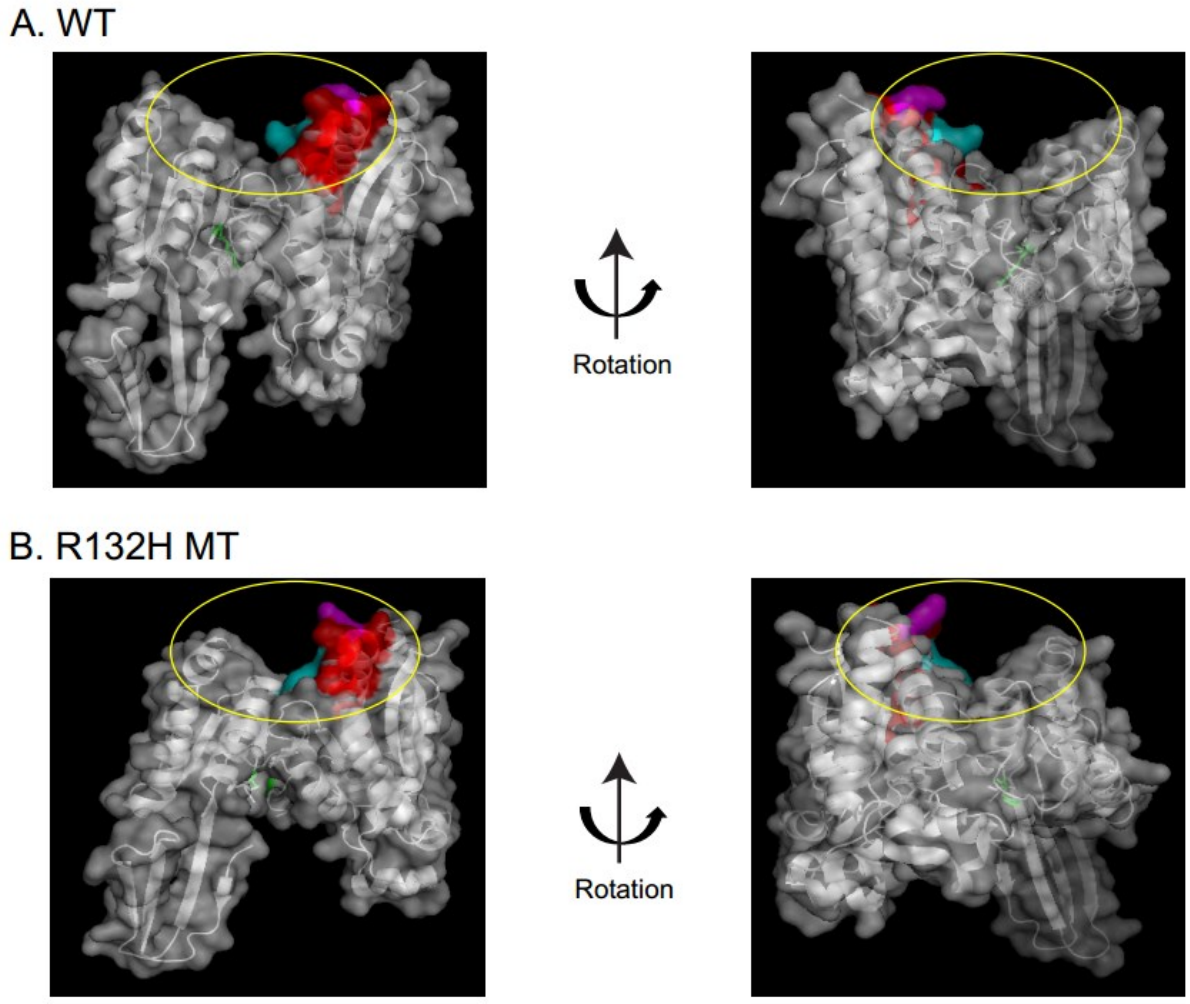

Figure 4. Comparison of the three dimensional structures of IDH1 WT (PDB ID: 1T0L) and R132H MT (PDB ID: 3MAP). IDH1 proteins are displayed with cartoon and surface. The peptide region for the CaM-binding is shown in red (amino acids; W336 ~ E352), magenta (K350), and cyan (K345). The structural difference of the cavity around the peptide region is indicated by yellow circles on IDH1 WT (A) and R132H MT (B). The amino acid residue 132 that is mutated in R132H is shown by green stick.

To further confirm the binding between CaM and IDH1 and to understand its meaning biologically, we investigated the effects of TFP, an established CaM antagonist. Conventionally, TFP has been used as an anti-psychotic drug, and some anti-psychotic drugs, such as TFP, thioridazine, and phenoxybenzamine, have been shown to inhibit growth of highly malignant glioblastoma cells *in vivo* and/or *in vitro* [18,26,27]. We obtained consistent results for the inhibitory effect of TFP on U87 glioblastoma cell survival and migration. We also showed that, in addition to these phenotypic effects, TFP actually interferes with the molecular interaction between CaM and IDH1 WT, providing a mechanistic insight in the TFP's anti-glioma activity. Proteins bound and regulated by CaM such as K-ras and CLC-3, have been known to be associated with glioblastoma migration and malignancy [13-17]. Furthermore, a CaM-dependent kinase II, CaMKK2, which functions as a signaling hub in diverse cellular regulatory pathways, is expressed differentially in IDH1 WT and MT glioma cells [22], suggesting that CaM signaling and binding proteins influence glioma malignancy and aggressiveness. The expression level of CaM seems also to be elevated in high-grade gliomas [10], though some studies have reported the opposite trend [18]. Still, it should be noted that inhibitions of CaM by an inhibitor or shRNA leads to reduction of migration and increased survival *in vivo* [10]. Considering all of these facts, we are tempted to speculate that IDH1 WT, but not IDH1 MT, may be a previously unknown target of CaM in high- grade gliomas, and that pharmacological disruption of CaM-IDH1 WT interaction may reduce the migration and aggressiveness of glioma cells. As the tumor-association of IDH1 MT has been found mostly in low-grade gliomas with better prognoses, there has even been a suggestion that IDH1 mutation might actually be a protective mechanism for overall patient survival [28]. More research into the roles of IDH WT in glioma tumorigenesis is warranted to determine if IDH mutation can act as a regulator of glioma aggressiveness by controlling the interaction between IDH and its binding proteins such as CaM.

We also obtained more detailed insight into how CaM interacts with IDH1 WT and how it can be disrupted by CaM-antagonist TFP. At a low stoichiometry (1:1), the IDH1 peptide induced chemical shifts only on residues at the C-lobe of CaM, especially those from M145 (1^{st} M) to A148 (see Fig. 2D and E). These residues coincide with the major binding residues for TFP at low stoichiometry, in the absence of calcium (M145 and M146 correspond to M144 and M145, respectively, in the original reference, due to a one-residue numbering difference) [29,30]. Therefore, TFP seems to directly compete with the IDH1 peptide at the same binding site on the C-lobe. At a higher stoichiometry (1:10), the IDH1 peptide induced much larger changes over residues in both the C- and N-lobes. Interestingly, up to four molecules of TFP can bind to CaM in the presence of calcium, and this higher stoichiometry interaction involves residues on both the C- and N-lobes [29]. The calcium-independent initial binding to the C-lobe and the subsequent binding to the N-lobe also has been shown for other CaM interactions [31,32]. The IDH1 peptide exhibited a similar stepwise binding mode, except that it did not require calcium for the N-lobe interaction. Instead, a higher concentration drove the interaction toward the two-lobe binding mode, which might represent another mechanism of CaM-partner interaction.

In conclusion, the identified new interaction between CaM and IDH1 WT, but not IDH1 MT, can be associated with the difference between high- and low-grade gliomas. Therefore, CaM's pharmacological inhibition could suggest a new strategy for the control of gliomas' aggressive properties.

Declaration of competing interest

None.

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This article contains Supporting Information (Methods, and Figures).

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